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RESEARCH ARTICLE

How to make a sexy snake: estrogen activation of female sex pheromone in male red-sided garter snakes

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SUMMARY

Vertebrates indicate their genetic sex to conspecifics using secondary sexual signals, and signal expression is often activated by sex hormones. Among vertebrate signaling modalities, the least is known about how hormones influence chemical signaling. Our study species, the red-sided garter snake (*Thamnophis sirtalis parietalis*), is a model vertebrate for studying hormonal control of chemical signals because males completely rely on the female sex pheromone to identify potential mates among thousands of individuals. How sex hormones can influence the expression of this crucial sexual signal is largely unknown. We created two groups of experimental males for the first experiment: Sham (blank implants) and E2 (17β-estradiol implants). E2 males were vigorously courted by wild males in outdoor bioassays, and in a Y-maze E2 pheromone trails were chosen by wild males over those of small females and were indistinguishable from large female trails. Biochemically, the E2 pheromone blend was similar to that of large females, and it differed significantly from Shams. For the second experiment, we implanted males with 17β-estradiol in 2007 but removed the implants the following year (2008; Removal). That same year, we implanted a new group of males with estrogen implants (Implant). Removal males were courted by wild males in 2008 (implant intact) but not in 2009 (removed). Total pheromone quantity and quality increased following estrogen treatment, and estrogen removal re-established male-typical pheromone blends. Thus, we have shown that estrogen activates the production of female pheromone in adult red-sided garter snakes. This is the first known study to quantify both behavioral and biochemical responses in chemical signaling following sex steroid treatment of reptiles in the activation/organization context. We propose that the homogametic sex (ZZ, male) may possess the same targets for activation of sexual signal production, and the absence of the activator (17β-estradiol in this case) underlies expression of the male phenotype.

Key words: activation, pheromone, chemical ecology, endocrinology, reproduction, snake.

INTRODUCTION

Secondary sexual signals (coloration, morphology, scents) mostly serve as honest indicators of an individual's genetic sex and potential mate quality. Many vertebrate secondary sexual signals are directly controlled by sex steroid hormones, which can reinforce their value as honest signals because primary sex steroids, such as testosterone and 17β-estradiol, exhibit sexually dimorphic patterns that correlate with an individual's genetic sex and quality/health. Such signals can be expressed seasonally (e.g. breeding plumage, skin coloration) or for the duration of the annual cycle (e.g. electric waveforms) and are often subject to modulation and/or regulation by sex steroids.

Sexual signals and the sensory systems required for their perception (visual, auditory, olfactory) are directly influenced by sex steroid hormones during development. Key experiments done by Phoenix et al. demonstrated that sex steroids are crucial for guiding the organization of neural centers controlling sex-specific behavior and that the presence of those same sex steroids or others are necessary later in life to activate sexual behavior controlled by these centers (Phoenix et al., 1959). Their results established that sex steroids have two major effects in vertebrate reproduction: permanent (organizational) and reversible (activational). Although this generalized verbal model has been adjusted to accommodate other, more complicated pathways through which sex hormones act (e.g. Moore et al., 1998), the concept of activational/organizational effects of steroids generally fits most systems and has repeatedly passed experimental tests [reviewed extensively in a special issue of *Hormones and Behavior* (Wallen, 2009)].

Organizational and activational effects of steroids on sexual signals (e.g. behavior, coloration) have been demonstrated in numerous vertebrate groups. For testing organizational effects, individuals or whole clusters of offspring are exposed either *in utero* or at birth to exogenous sex steroids and then followed into adulthood to examine the effects of treatment on specific secondary sexual signals. Largely, these experiments study changes in behaviors and their neurological centers following steroid treatment [e.g. singing behavior in zebra finches, *Taeniopygia guttata* (Gurney and Konishi, 1980) and courtship behavior in Japanese quail, *Coturnix japonica* (Adkins, 1979)]. Activational effects of steroid hormones on secondary sexual signals, however, focus on the signals themselves and how they respond to steroid treatment over smaller windows of time, typically in adults. The activation of conspicuous and/or powerful sexual signals by primary sex steroids is present in all vertebrate groups [e.g. fish (Liley and Stacey, 1983), amphibians (Kelley and Pfaff, 1976), reptiles (Cooper et al., 1987), birds (van Oordt and Junge, 1934) and mammals (Carlisle et al., 1981)].

An insightful experimental approach to understanding the organizational or activational effects of sex steroids is the reversal of sexual signal expression. Many studies have demonstrated organizational reversal of secondary sexual characteristics, especially experiments on endocrine disrupting compounds. For example, female mosquitofish (*Gambusia affinis holbrooki*) exposed to metabolites of dichlorodiphenyltrichloroethane (DDT) express male characteristics, such as gonopodia and courtship behavior (Howell et al., 1980), and male alligators (*Alligator mississippiensis*) become feminized following exposure to the same compounds in the same area in Florida [Lake Apopka, FL, USA (Guillette et al., 1996; Guillette et al., 1999)]. Of particular interest are studies where the reversal of signal production is purely activational. A trend among these studies is the treatment of females with androgens, which typically results in females exhibiting the male signal [e.g. waveforms in electric fish (Dulka and Maler, 1994), calling behavior in frogs (Penna et al., 1992), morphology in anoles (Lovern et al., 2004), plumage in ruffs (Lank et al., 1999) and mounting behavior in rodents (Phoenix et al., 1959; Goldfoot and van der Werff, 1975).]

Although classic sexual signals (e.g. calling behavior, plumage) are well characterized in terms of the activation/organization context in vertebrate endocrinology, chemical cues have not been evaluated with the same rigor despite the fact that they are equally powerful, specific signals. There are examples in vertebrates of skin lipids and glandular lipids changing following sex steroid treatment but these studies did not have biochemically identified sexual attractants that could be quantified and tested in behavioral assays (e.g. plateau lizards (Abell, 1998) and hamsters (Takayasu and Adachi, 1970; Lutsky et al., 1975). Our species, the red-sided garter snake, *Thamnophis sirtalis parietalis*, is an exceptional model for understanding the relationship between sex hormones and chemical signal production.

The red-sided garter snake utilizes a potent chemical signal, the female sexual attractiveness pheromone, to identify and choose between mates in massive spring mating aggregations (>10,000 individuals) in the Interlake Region of Manitoba, Canada. The composition of the pheromone relays several key pieces of information to males: species (Mason, 1993), population (LeMaster and Mason, 2003), sex (Mason et al., 1989), season (Mason et al., 1987; LeMaster and Mason, 2001; Parker and Mason, 2009), reproductive condition (Shine et al., 2003), and age (LeMaster and Mason, 2002). More interesting is that male red-sided garter snakes can be deceived into courting other males that naturally produce small amounts of female sex pheromone ('she-males'). She-males appear to gain a thermoregulatory advantage during spring mating (Mason and Crews, 1986; Shine et al., 2001). Thus, there are examples of both honest and dishonest signaling *via* sex pheromones in the red-sided garter snake system, which parallels findings from another group of reptiles (Lacertid lizards) demonstrating condition-dependent sexual signaling *via* skin lipid secretions (López and Martín, 2005; López et al., 2006; Martín and López, 2008).

The female sexual attractiveness pheromone of the red-sided garter snake is a blend of non-volatile, long-chain (C_{29}–C_{37}) saturated and monounsaturated methyl ketones ranging from 394 Da to 532 Da (Mason et al., 1989). Individual pheromone components, when presented singly, elicit much lower levels of courtship from males compared with the complete blend; however, the longest, unsaturated components can elicit significant courtship behavior when presented alone (Mason et al., 1989). We can quantify the relative contributions of each methyl ketone in the profile in addition to examining the ratio of saturated to unsaturated molecules. We can then determine how other factors (e.g. snake length, body condition) explain the differences quantified in pheromone profiles. These comparisons illuminate differences that arise in bioassays [i.e. explaining why longer, larger females are preferred by males in courtship trials (LeMaster and Mason, 2002; Shine et al., 2003)]. Although much is known about the quantitative and qualitative aspects of the pheromone that controls male mate choice in our system, only one study has successfully examined the effects of a sex steroid (17β-estradiol) on female attractivity [*via* ovariectomy/hormone replacement (Mendonça and Crews, 1996)]. Although the results in that study were confounded by incomplete ovariectomy in some of the experimental animals, they determined that 17β-estradiol is the sex steroid that maintains female attractivity (Mendonça and Crews, 1996).

This study is comprised of two experiments. In the first experiment, we implanted male red-sided garter snakes with 17β-estradiol implants to: (1) assess whether estrogen can make males attractive, and (2) quantify the changes in lipid composition/pheromone production induced by estrogen treatment. For the second experiment, we followed the same procedures as the first but removed estrogen implants in the second year to determine whether estrogen's effect on attractivity and pheromone production is activational or organizational.

MATERIALS AND METHODS

Animals and treatment groups

Red-sided garter snakes (*Thamnophis sirtalis parietalis* Say in James 1832) emerge in the tens of thousands every spring from concentrated limestone hibernacula in the Interlake Region of Manitoba, Canada. Upon emergence, adult males engage in an intense scramble competition for access to females, who emerge singly and sporadically over the course of the mating season [April–May (Gregory, 1974)]. We collected courting male snakes (N=20) in the spring of 2006, brought them to the laboratory at Oregon State University, OR, USA, and created two groups of experimental males (N=10 each): Sham (no implant) and E2 (17β-estradiol implant) for the first experiment.

For the second experiment, we collected courting males in the spring of 2007 (N=16) and the spring of 2008 (N=8). Three groups were created: Sham (blank implant given in 2007), Removal (17β-estradiol implant given in 2007, removed in 2008), and Implant (17β-estradiol implant given in 2008). The Sham and Removal snakes were taken to the field for bioassays in May 2008 and 2009. Implant snakes were collected in 2008 and taken back to the field in 2009. All males fell within a set body length range (45–55 cm snout-to-vent length), and there were no significant differences in length between groups in either experiment.

Snakes were kept in the laboratory under simulated summer conditions for all summers [26°C:18°C, 16 h:8 h light:dark (L:D)] and fed a mixed diet of earthworms and salmon smolt. Water was provided *ad libitum*. They were then hibernated for six months in the laboratory over the winters (4°C, 0 h:24 h L:D) and always taken back to the site of capture in Inwood, Manitoba, Canada, in spring (April) for behavioral testing.

Surgical procedures

Snakes were anaesthetized with a subcutaneous injection of brevital sodium (0.003 ml of 0.5% solution per 1 g body mass) until righting reflex was abolished [~15 min (see Wang et al., 1977)]. Sterile corneoscleral scissors were used to make the incision between the second and third dorsal scale rows about two-thirds down the length of the animal (roughly positioned next to the right testis). Silastic implants (0.033 mm i.d. × 0.05 mm o.d. × 5 mm length; Dow

Corning, Midland, MI, USA) were created from tubing sealed with medical adhesive at both ends after either being filled with crystalline estradiol (17β-estradiol, Sigma Chemical Co., St Louis, MO, USA) or left empty (Sham). Implants were inserted into the peritoneal cavity. Following surgery, the incision site was sutured (4-0 silk suture fitted to a small cutting needle) and the snakes were allowed time (6 h) to recover in a sterile cage before being placed back into their home cage. All procedures involving the use of live animals were approved by the Institutional Animal Care and Use Committee at Oregon State University (ACUP 3120) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The collection and use of these animals was approved by Manitoba Conservation (Manitoba Wildlife Scientific Permit WB02024).

Behavioral tests

Arena trials

For the first experiment, the first set of behavioral tests were done in cubical outdoor arenas {1 ×1×1 m, *N*=4 [for an example, see Shine et al. (Shine et al., 2000)]} at the den site in Inwood, Manitoba, Canada, in 2007. Individual experimental males were randomly selected, uniquely marked on their heads with a silver paintpen (Sharpie, Oak Brook, IL, USA), and placed individually into an arena. Courting wild males (*N*=10) were then placed into each arena, and the proportion of males courting the experimental male was recorded after 10 min by an observer who was blind to the experimental treatments. Previous work has shown that male red-sided garter snakes exhibit facilitated courtship, where larger groups of competing males induce more vigorous, reliable male courtship behavior in arena trials than assays using single males paired with a female (Joy and Crews, 1985). Courting males were distinguished by conspicuous chin-rubbing behavior where the male tongue-flicks the dorsal surface of the female and aligns his body with hers, a behavior seen only in the reproductive context [see Moore et al. (Moore et al., 2000) and LeMaster (LeMaster, 2002) for recent ethograms describing male red-sided garter snake reproductive behavior]. Arena trials were only conducted for the first experiment and not for the second.

Mating ball tests

Mating balls composed of several males courting individual females serve as ultimate scenarios for assessing the attractiveness of experimental animals. Previous work in this system tested male mating behavior and female attractiveness in outdoor enclosures [(e.g. Shine and Mason, 2001) described above]. Although these outdoor arenas are ideal for measuring male courtship behavior directed toward females, experimental males (the target animals of this study) do not permit/tolerate courtship for long periods of time and will actively avoid courtship from other males (M.R.P., personal observation). To overcome this difficulty in assessing experimental male attractiveness, we developed a bioassay where mating balls containing a single female were started in the den by placing a newly emerged female on the ground until ~20 males began to court her. The female had her cloaca taped to prevent copulation because mated females rapidly become unattractive (Devine, 1977). The taping procedure does not affect male or female behavior (LeMaster and Mason, 2002; Lutterschmidt et al., 2004). Once the mating ball formed, we introduced the experimental animal within 20 cm of the mating ball. An experimentally blind observer counted the number of males that left the mating ball to court the stimulus male over a 1 min period without replacement. Wild males were removed from the experimental male if they exhibited chin-rubbing behavior [a score of 3 in the ethogram of Moore et al. (Moore et al., 2000)]. The reliability of this bioassay was equal to the arena trials. The mating ball behavioral test was used for both experiments.

Y-maze trials

We used a Y-maze (1 m, each arm) to determine the capacity of the E2-induced pheromone in eliciting trailing behavior in wild male snakes following a previously published methodology (see LeMaster et al., 2001). In our tests, we used novel, courting males and tested their abilities to choose between paired chemical trails that spanned the length of the base and one entire arm of the maze. The first test was a bias test where both arms were without scent (blank). The remaining trials all paired an E2 male's pheromone trail with the trail from: Sham males, she-males, small females (60 cm), and large females (75 cm; the last three were all caught on-site at the den). In brief, a male was acclimated in a hidebox until it chose to leave the box and encountered the chemical trail (usually within 5 min). The pheromone trail was created by rubbing the lateral surface of a snake along the paper substrate and pegs (covered in disposable straws) in the Y-maze in a weaving pattern [depicted in Parker and Mason (Parker and Mason, 2011)]. The two scents were crossed at the junction of the Y to ensure that trailing males were forced to make a decision at the junction (LeMaster et al., 2001). Males were said to have made a choice for an arm once their head passed the last set of pegs in an arm (90 cm from the junction of the Y). The Y-maze was only used for the first experiment.

Chemical analyses

17β-estradiol

Blood (0.3 ml) was taken from all males *via* the caudal vein within 1 min of restraint using heparinized syringes (1 cm^3, 25 g). Blood was stored on ice (<4 h) until centrifugation to separate out red blood cells (100 ***g***, 5 min), and the plasma was frozen at –20°C and stored at –80°C until used in direct radioimmunoassay following modified procedures of Lutterschmidt et al. (Lutterschmidt et al., 2004) and similar to previous hormone studies (e.g. Whittier et al., 1987).

Briefly, we extracted steroids from plasma aliquots (20–40 µl) with anhydrous ethyl ether. The ether phase was isolated and dried under nitrogen gas in a water bath (35°C). Hormone extracts were resuspended in phosphate-buffered saline and incubated with tritiated estradiol (2,4,6,7,16,17-3H estradiol; GE Healthcare/Amersham Biosciences, Piscataway, NJ, USA) and estradiol antiserum (Fitzgerald Industries International, Concord, MA, USA) at 4°C for 12–24 h. Cross-reactivity of this antiserum with estrone is 1.0%; cross-reactivity with estriol is <0.2%; cross-reactivity with testosterone is <0.1%. Unbound steroid was separated from bound hormone using dextran-coated charcoal (Sigma Chemical Co.), and the radioactivity of each sample was quantified in a Beckman SC 6000 scintillation counter (Greer, SC, USA). Samples were assayed in duplicate and corrected for individual recovery variation. Mean extraction efficiency for 17β-estradiol was 99%, as determined by the recovery of tritiated estradiol added to samples before extraction with ethyl ether. All samples (i.e. treatment groups) were randomly distributed within the assay.

Implantation resulted in a mean 17β-estradiol concentration of 0.28 ng ml^{-1} for both E2 and Implant males. Sham males in both experiments had undetectable 17β-estradiol levels. In the second experiment, Removal males had undetectable 17β-estradiol levels in 2009 compared with Implant males (Mann–Whitney, U=6.00, P<0.001). Mean intra-assay variation was 7.3%, interassay variation was 14.1%, and the mean limit of detection was 14 pg ml^{-1}. Previous work on red-sided garter snakes found that estrogen treatment

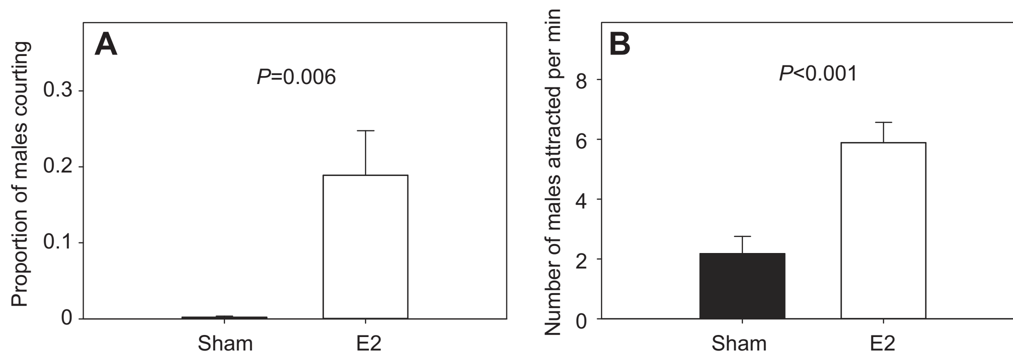

Fig. 1. Results from bioassays of male courtship behavior directed toward experimental males. (A) Proportion of males (*N* out of 10; mean + s.e.m.) courting the stimulus male in arena trials after 10 min. (B) Mean number of males (+ s.e.m.) attracted from a mating ball by the experimental male. Sham males received surgery only whereas E2 males received an 17β-estradiol implant.

(intraperitoneal injections) in males increased liver mass and blood viscosity (Garstka and Crews, 1981). As further validation for the estrogen implants, we measured the liver mass of E2 males and Shams in Experiment One. E2 liver mass was twice that of Shams (0.67 g *vs* 0.38 g; t=5.94, P<0.001), and vitellogenin production might have increased in E2 snakes, as evidenced by an increased viscosity of blood plasma. The 17β-estradiol implants in this study resulted in lower levels of 17β-estradiol than those experienced naturally by females in the field [5.5–6.5 ng ml^{-1} post-mating; 0.8–1.0 ng ml^{-1} during vitellogenesis (Whittier et al., 1987)].

Pheromones

All snakes were euthanized at the end of each experiment for pheromone collection following the methods of Mason et al. (Mason et al., 1989). Snakes were euthanized with a lethal overdose of brevital sodium (6 mg kg^{-1}) prior to individual collection of skin lipids by immersion in hexane for 12 h. The volume of the skin lipid extracts was reduced under vacuum using a rotary evaporator, and the total skin lipid yield of the dry product was determined (mg) before fractionation. The pheromone was isolated using alumina columns [activity III (Sigma Chemical Co.); pooled fractions 4-6 (2% diethyl ether:98% hexane as mobile phase)]. The pooled fractions containing pheromone were reduced by using a rotary evaporator and weighed to determine the mass (mg). The combined pheromone fractions are composed almost solely of methyl ketones that comprise the red-sided garter snake sexual attractiveness pheromone (>99% of fraction mass). The pooled pheromone fraction was resuspended in a pheromone:hexane mixture (1 mg:1 ml) before analysis with gas chromatography/mass spectrometry (GC/MS).

Individual pheromone samples were analyzed with a Hewlett Packard (HP) 5890 Series II gas chromatograph fitted with a split injector (280°C) and an HP 5971 Series mass selective detector (Palo Alto, CA, USA). Aliquots (1 μl) of the 1:1 samples (1 mg pheromone:1 ml hexane) were injected onto the fused-silica capillary column (RTX-1; 15 m × 0.25 mm i.d.; Restek Corporation, Bellafonte, PA, USA) with helium as the carrier gas (5 cm s^{-1}). All injections were made in the splitless mode with the split valve closed for 60 s. Oven temperature was held initially at 70°C for 1 min, increased to 210°C at 30°C min^{-1}, held at 210°C for 1 min, increased to 310°C at 5°C min^{-1}, and held at 310°C for 5 min. Individual compounds were identified using mass spectral data and ion chromatograms comparing our spectra with published data and authentic standards (Mason et al., 1990). By using the peak integration function in ChemStation software (Agilent, Santa Clara, CA, USA) interfaced with the GC/MS, we determined the relative contributions of each component of the pheromone to the overall profile of each snake. By using an internal standard [methyl stearate, 20 μg ml^{-1} hexane (LeMaster et al., 2008)], we were able to derive individual component mass (μg) for all of the 17 methyl ketones comprising the pheromone for the second experiment.

Statistical analyses

For behavioral data (arena trials, mating ball tests), we used either *t*-tests (Experiment One) or one-way non-parametric ANOVAs (Kruskal–Wallis) with treatment as the factor (α=0.05) followed by multiple comparisons (Dunn's test; SigmaStat 3.1, Systat Software Inc., Point Richmond, CA, USA) for Experiment Two, because that experiment had three experimental groups. We used binomial tests to analyze the choice test data from the Y-maze trials in Experiment One. Pheromone profiles were analyzed with the Multi Response Permutation Procedure (MRPP) followed by Bray–Curtis analysis using Sorenson distance for multiple comparisons [R statistical computing software (McCune et al., 2002)]. This method for pheromone analysis was also used in previous publications (Parker and Mason, 2009; Mason and Parker, 2010). We also generated the coordinates for non-metric multidimensional scaling (NMS) plots using the vegan package in R. All graphics were produced with SigmaPlot 8.0 (SysStat Software, Inc.).

RESULTS

Behavioral trials – Experiment One

Estrogen implantation caused male snakes to become attractive in both experiments. In the arena trials and the mating ball tests for Experiment One, E2 males were courted more and attracted more males than Sham snakes (Mann–Whitney, U=70.0, P=0.006; t=4.227, P<0.001, respectively) (Fig. 1A,B). In the Y-maze trials, wild male red-sided garter snakes chose the arm containing the E2 scent trail more times than the scent of Sham snakes (Z=2.88, P<0.001), natural she-males (Z=2.31, P=0.01) and small females (Z=2.31, P=0.01; Fig. 2A). Trailing males tended to choose the large female pheromone trail over the E2 trail but this was not significant (Z=1.09, P=0.09).

Pheromone analyses – Experiment One

The quality of the red-sided garter snake sex pheromone is detected by males as a mixture of compounds, not as individual components; thus, we analyzed the total chemical composition of the pheromone profiles for each experimental animal. E2 males differed from Sham males in their total pheromone compositions, both quantitatively (A=0.2876, δ=0.2506, P<0.001; Fig. 2B) and qualitatively (Fig. 2C). When female pheromone extract data from another study (Parker and Mason, 2009) were added to the analysis, all three groups (E2, Sham, Female) were significantly different from one another (E2 *vs* Female, A=0.1096, δ=0.2391, P=0.002; E2 *vs* Sham, A=0.1575,

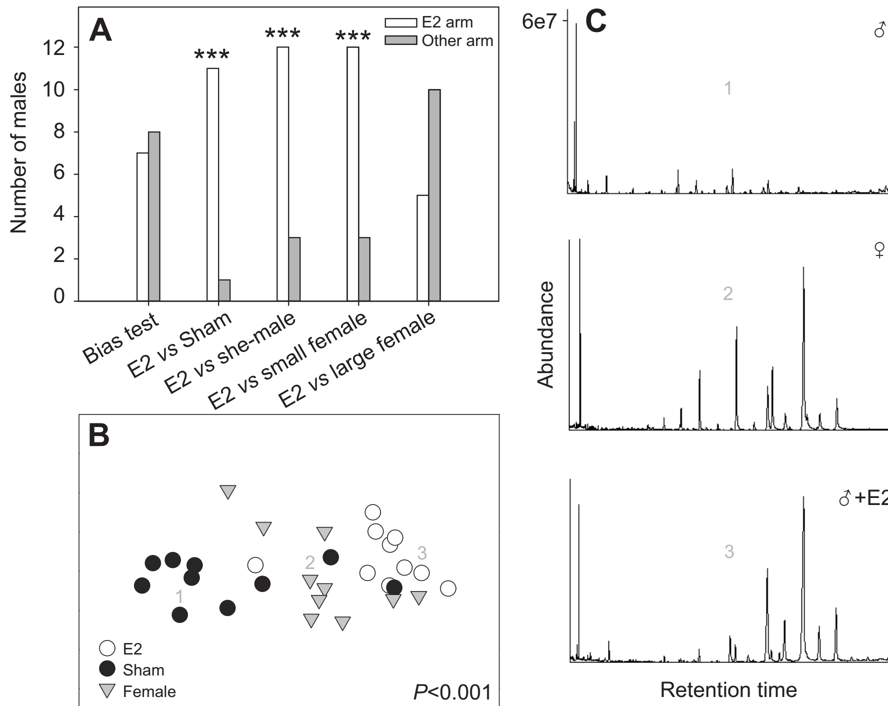

Fig. 2. (A) Results of Y-maze experiments with wild males following scent trails. White bars represent the number of unique, courting males that chose the E2 male scent trail over the stimulus presented in the other arm (gray bars). The bias test presented males with no stimulus scents. Asterisks represent statistically significant choice for an arm ($P<0.05$). (B) Non-metric multidimensional scaling (NMS) plot depicting differences in pheromone composition between the groups. Female profiles are plotted only for visualization purposes. Points close together represent individual snakes with more similar pheromone blends. Stress=5.61. The numbers 1–3 indicate which individual GC traces were chosen for qualitative representations for C. (C) Gas chromatograph traces from three (1–3) representative animals from the NMS plot. Each trace is scaled to the same molecular abundance (6e7). The large peak coming off first is the methyl stearate standard used to obtain concentration values for each methyl ketone in the pheromone profile.

δ=0.2163, P=0.001). The stress for the NMS plot was 5.61. There is interesting variability within groups, especially among females, and this corroborates findings from other studies on the composition of the red-sided garter snake sex pheromone (e.g. LeMaster and Mason, 2002; Parker and Mason, 2009).

Previous work has shown that pheromone profiles can differ in their ratios of unsaturated (U) to saturated (S) methyl ketones (U:S) and that males rely on this ratio to make mate choice decisions (LeMaster and Mason, 2002; Parker and Mason, 2009). We analyzed the U:S ratios for E2 *vs* Sham males and found that E2 males had much greater U:S ratios than Sham males (Mann–Whitney, U=98.0, P<0.001; Fig. 3A). The U:S ratios differed by almost an order of magnitude between the two groups (0.98 for Sham; 9.02 for E2). The methyl ketones comprising the red-sided garter snake sex pheromone can be separated into two size classes: low molecular mass (<463 Da; 'L') and high molecular mass [>463 Da; 'H' (Parker and Mason, 2009)]. Previous work has shown that larger females have pheromone blends dominated by unsaturated methyl ketones that fall mostly in the H size class we are describing (LeMaster and Mason, 2002). From our analysis in the current experiment, we found that E2 males had a heavier pheromone composition (higher H:L) than Sham males (Mann–Whitney, U=93.0, P=0.001; Fig. 3B).

Behavioral trials – Experiment Two

Between 2008 and 2009, Sham males did not change in their attractiveness (Wilcoxon signed rank test, W=0.00, P=0.841), so we conducted a one-way ANOVA on the 2009 bioassay data using only the 2009 Sham data. There was a significant treatment effect ($F_{2,23}$=74.330, P<0.001), and Implant males were more attractive than both Sham and Removal males (Tukey's test, q=16.050, P<0.001; q=13.815, P<0.001, respectively; Fig. 4A). Sham and Removal males did not differ in their attractiveness (q=1.487, P>0.05). Removal males were more attractive in 2008 when they had their estrogen implants than in 2009 after the implant had been removed (paired t-test, t=3.721, P=0.010; Fig. 4B).

Pheromone analyses – Experiment Two

Pheromone collection is terminal so only pheromone extracts could be collected and analyzed in 2009. Treatment had an effect on total pheromone mass (mg; $F_{2,25}$=12.768, P<0.001), and Implant males produced more pheromone than both Sham and Removal males (q=6.887, P<0.001; q=5.251, P=0.003, respectively). Total pheromone mass (mg) was not different between Sham and Removal males (q=1.352, P>0.05). When all 17 methyl ketones were analyzed for total abundance with MRPP in R, there was an overall difference in composition between the three groups (A=0.2088, δ=0.3998, P<0.001; Fig. 5). Implant males had pheromone blends that were distinct from Sham males (A=0.2943, δ=0.3629, P<0.001) and Removal males (A=0.1932, δ=0.4248, P=0.005). Sham and Removal males did not have different pheromone blends (A=–0.0171, δ=0.4145, P>0.05). Stress for the NMS plot was 11.32.

The individual components of the pheromone blends were analyzed *via* a two-way ANOVA [methyl ketone molecular mass (Da), treatment] to determine which components were affected by estrogen treatment. Treatment and methyl ketone molecular mass both had significant effects on methyl ketone mass produced by snakes, and there was a significant interaction ($F_{2,25}$=5.452, P=0.012; $F_{16,25}$=106.645, P<0.001; $F_{32,46}$=8.760, P<0.001, respectively). The methyl ketones weighing 476, 490, 504, 518 and 532 Da, all unsaturated compounds within the pheromone blend, were found in higher amounts in Implant males than Sham males (q=6.914, P<0.001; q=7.599, P<0.001; q=11.478, P<0.001; q=9.587, P<0.001; q=10.008, P<0.001, respectively) and Removal males (q=5.421, P<0.001; q=6.563, P<0.001; q=9.202, P<0.001; q=7.316, P<0.001; q=6.769, P<0.001, respectively; Fig. 6A). Interestingly, the only methyl ketone that was found in higher amounts in Removal males than in Sham males was the 532 Da compound (q=2.873, P=0.045). There was an overall difference in the U:S ratios among the three groups ($F_{2,25}$=22.454, P<0.001), and Implant males had higher ratios than both Sham and Removal males (q=9.044, P<0.001; q=7.173, P<0.001, respectively). Sham and Removal males did not differ in their U:S ratios (q=1.483, P>0.05; Fig. 6B).

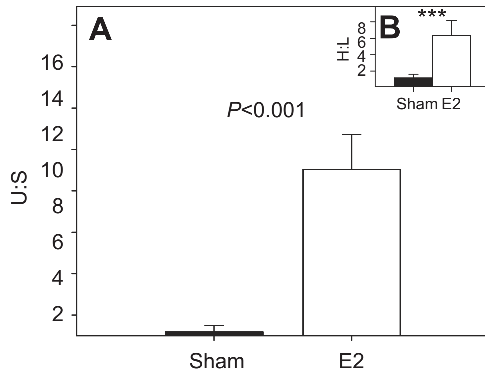

Fig. 3. (A) Ratio of unsaturated (U) to saturated (S) pheromone components (methyl ketones) in male red-sided garter snake skin lipids. Estrogen-implanted males (E2) had pheromone blends consisting mostly of unsaturated methyl ketones. (B) Ratio of high molecular mass (H) methyl ketones to low molecular mass (L) methyl ketones in male skin lipids (P=0.001). Bars are means + s.e.m.

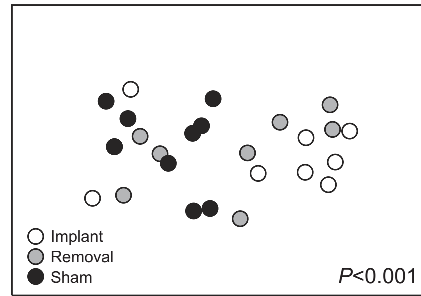

Fig. 5. Non-metric multidimensional scaling (NMS) plot derived from relative proportions of the 17 unique methyl ketones comprising the pheromone blends from the three groups. Implant males had significantly different pheromones than both Sham and Removal males but the latter two were not different. Stress=11.32.

DISCUSSION

Estrogen treatment resulted in the production of female pheromone in male red-sided garter snakes, and this pheromone elicited intense courtship behavior by wild males in the den during the mating season. The estrogen-induced pheromone is composed primarily of long-chain, unsaturated methyl ketones of the same length and structure naturally produced by large female red-sided garter snakes in the spring in the field (LeMaster and Mason, 2002). More interestingly, only specific components of the red-sided garter snake sex pheromone were increased following estrogen treatment, namely the methyl ketones weighing 476, 490, 504, 518 and 532 Da (Fig. 6). These methyl ketones are all unsaturated molecules with a double bond in the ω-9 position (Mason et al., 1989), suggesting that estrogen specifically initiates the production of (only) these methyl ketones.

The hormonal dependence of pheromone production has been established behaviorally or quantitatively in other vertebrates [e.g. fish (Liley and Stacey, 1983), amphibians (Kikuyama et al., 2005), reptiles (Cooper et al., 1986), birds (Bohnet et al., 1991) and mammals (Iwata et al., 2000)]. These and related studies suggest their observations support activational effects of steroids on pheromone production given the cessation of pheromone action/expression in the absence of the steroids in question. However, the true test of the activational effects of steroid hormones requires a combination of bioassays along with quantitative and qualitative assessment of changes in the specific signal itself as well as the steroid(s) in question, as is the case in our study. To our knowledge, this is the first study in a vertebrate that has specifically quantified hormone-induced changes in a chemical signal eliciting consistent, robust reproductive behaviors in the natural environment.

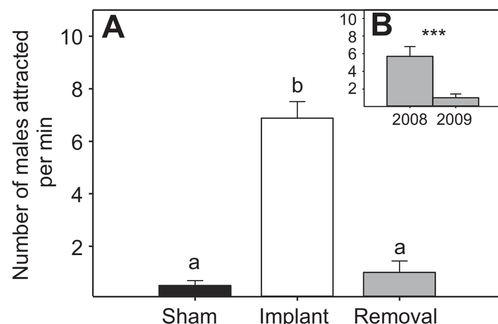

Fig. 4. Mean number of males (+ s.e.m.) attracted from a mating ball by experimental males in both 2008 (B) and 2009 (A). (A) Experimental males implanted with estrogen (Implant) were more attractive than Sham males and old estrogen-implanted males whose implants had been removed (Removal). (B) Removal males were only attractive in 2008 when they had circulating levels of estrogen, an effect that was abolished by implant removal in 2009. Asterisks or different letters above bars represent statistical differences (P<0.05).

Short-term increases in attractivity or courtship behavior can be induced in reptiles *via* steroid injections (17β-estradiol and testosterone, respectively) but the reliability of estrogen injections to increase attractivity and/or receptivity in female reptiles is highly variable [summarized in Mason (Mason, 1992) and Whittier and Tokarz (Whittier and Tokarz, 1992)]. Such paradoxical results might be explained by the susceptibility of the skin to steroid treatments during different phases of the shedding cycle. The skin of female red-sided garter snakes is sensitive to the feminizing effects of 17β-estradiol only if the injections are given more than five days before shedding (Kubie et al., 1978). Thus, the skin that is exposed after shedding can only be affected by steroid treatment if the treatment is administered at the appropriate time. In our study, the red-sided garter snakes were implanted for nine months with small, non-lethal steroid implants that constantly provided the skin with physiological levels of 17β-estradiol, which is likely to be the reason why we were successful in inducing males to produce female pheromone and then assess the quality of this pheromone in field bioassays.

Our study has elucidated two points concerning the relationship between gonadal sex steroid hormones and sexual signals in red-sided garter snakes. One is that pheromone production is directly related to estrogen signaling (e.g. Mendonça and Crews, 1996). Males implanted with estrogen produced a pheromone very similar in composition to that of large females. In bioassays, the pheromone profile produced by estrogen-treated males was indistinguishable from that of wild females and elicited significant courtship behavior from wild males (Fig. 1A,B and Fig. 4). These lines of evidence suggest that female pheromone production and expression is at least initiated, if not maintained, by estrogen.

Many species of reptiles and even whole groups of reptiles (e.g. snakes) rely heavily/solely on chemical information when making mate choice decisions (for reviews, see Mason, 1992; Mason and Parker, 2010). Behavioral evidence for the importance of sex steroids in the regulation of sexually dimorphic chemical signals in reptiles

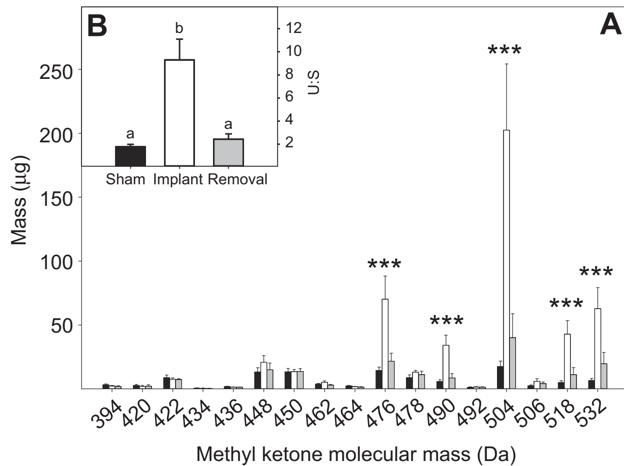

Fig. 6. (A) Mass of methyl ketones (µg; mean + s.e.m.) for each of the 17 unique methyl ketones [classified by molecular mass (Da)] between the three groups. Only the methyl ketones weighing 476, 490, 504, 518 and 532 Da were significantly higher ($P<0.05$) in estrogen-implanted males (Implant) than Sham and Removal males (asterisks). Removal males produced more of the 532 Da methyl ketone than Sham. (B) Ratios (mean + s.e.m.) of unsaturated:saturated (U:S) methyl ketone molecules in the pheromone blends of male red-sided garter snakes. Different letters represent statistical differences ($P<0.05$) between groups.

has only clearly been shown in one other species. A study in leopard geckos, *Eublepharis macularius*, showed that chemical cues from androgen-treated females elicited aggressive reactions from normal males (Rhen and Crews, 2000). Female geckos in that study were progressively masculinized by castration, androgen implantation, and finally by combining castration and androgen implantation. Work preceding that of Rhen and Crews (Rhen and Crews, 2000) demonstrated that chemical recognition by conspecifics in the same species of gecko is dependent on the shedding cycle (Mason and Gutzke, 1990). In that study, male leopard geckos acted aggressively to shedding females but engaged in courtship behavior with the same females once their new skin was accessible for chemical sampling (licking behavior). Taken together, the data from these previous studies corroborate our findings and interpretation while also raising an interesting question about how castration may affect pheromone production in the red-sided garter snake system. We have recently shown that, qualitatively, castration of males induces a female-typical pheromone (Parker and Mason, 2011). We also have evidence that testosterone plays a key role in regulating pheromone production (M.R.P. and R.T.M., unpublished data).

The second major finding from our study is that the pheromone production by estrogen-implanted males is reversible, suggesting estrogen activates the synthesis of female sex pheromone in adult snakes. Removal males returned to expressing typical male pheromone blends with reduced pheromone quantity and low U:S ratios (Figs 5 and 6). It may be that female red-sided garter snakes, which are larger than males in our system, elicit courtship because of the combined effect of quantitative and qualitative differences in the blend of methyl ketones present in the skin. Both sexes produce methyl ketones but males may have adapted to respond to differences in concentration (‘quantity’) and blend (‘quality’) of the methyl ketones that serve as sexual signals in this system. Because the skin of male red-sided garter snakes can be signaled to produce female pheromone by estrogen treatment, it raises a question about how genetic sex is expressed by males. In most species of reptiles, males are the homogametic sex (ZZ) and produce greater levels of circulating androgens than do females. Also, male reptiles typically have negligible circulating 17β-estradiol. Based on the evidence we have presented in this paper on the red-sided garter snake, we propose that the lack of 17β-estradiol signaling to the skin is what establishes the sexual dimorphism in pheromone composition. Thus, the skin of a snake is capable of synthesizing female sex pheromone but it must be signaled to do so. This conclusion has major implications for how endocrine disrupting compounds, such as estrogen-mimicking pollutants, may be of specific concern for species relying on chemical signals for successful reproduction. Further, our findings reveal that a major signaling molecule, 17β-estradiol, directly regulates the expression of a potent sexual signal that may exist in all snakes much in the same way diet-derived chemicals (e.g. vitamin E) regulate sex-specific skin lipids necessary for mate choice in lizards [*Lacerta viridis* (Martín and López, 2010; Kopena et al., 2011)].

The relationship between attractivity and 17β-estradiol in female red-sided garter snakes is perplexing. While female red-sided garter snakes have reduced/diminished attractivity following ovariectomy (Mendonca and Crews, 1996), a female cannot be rendered completely unattractive *via* ovariectomy. Indeed, Ovex females in the aforementioned study still elicited some male courtship in laboratory behavioral trials. This does not occur with Sham *vs* Ovex females when tested in the field (M.R.P., personal observation), suggesting laboratory and field assessments of attractivity may not be comparable. Ovariectomy, while reducing activational levels of estrogen, does not completely block 17β-estradiol production. Also, treatment with tamoxifen, a non-specific estrogen receptor antagonist, and ovariectomy fail to reduce/diminish attractivity in female red-sided garter snakes (M.R.P., unpublished data). These results have led us to suggest that the skin of females is organized to produce the sex pheromone early in life whereas male skin has the activational capacity for producing sex pheromone. Male reptiles and their skin secretions do not normally elicit courtship behavior from other males but she-male red-sided garter snakes in the Manitoba population serve as an illuminating exception (Mason and Crews, 1985). These males produce appreciable amounts of female pheromone but the hormonal mechanisms underlying this natural phenomenon remain elusive (Mason and Crews, 1986). There may be specific differences in the biology of she-male *vs* male red-sided garter snake skin, especially in the activational steroid that may be acting on their skin to signal pheromone production. Experiments using hormonal manipulation of she-males and normal males would reveal the proximate mechanisms regulating secondary sexual signal production in this and, by proxy, all snake species.

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